

### Tumour vaccines

5 The invention relates to the field of the immunotherapy of tumoral diseases. The majority of malignant tumours successfully escape control by the immune system, even though a number of them express tumour-associated antigens (van der Bruggen, 1991; Brichard, 1993; 10 Gaugher, 1994; Coulie, 1994). It has been found that, in addition to the presentation of the appropriate antigens, certain adjuvants are also needed in order to achieve effective stimulation of the immune system (Zier, 1995).

15 Early studies were aimed at stimulating the immune system by vaccinating with inactivated tumour cells in conjunction with non-specific immunostimulators such as BCG or other bacterial adjuvants and in some cases led 20 to an improvement of the survival rate of patients with malignant tumours (Berd, 1990; Barth, 1994; Morton, 1992). The effect of these adjuvants is not direct, but proceeds via the excretion of a cascade of endogenous cytokines and mediators and is therefore non-specific, 25 on the one hand, and hardly reproducible, on the other. This makes it difficult to use these non-specific adjuvants in a pharmaceutical composition.

In modern approaches, the immunostimulant cytokines are 30 used directly as adjuvants (Rosenberg, 1988, Rosenberg, 1989). Of the immunostimulant cytokines, interleukin-2 (IL-2), interferon- $\alpha$  (IFN- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), IL-12 and GM-CSF (granulocyte macrophage stimulating factor) have proved to be particularly promising (Rosenberg, 35 1991; Dranoff, 1993; Zatloukal, 1993; Ferrantini, 1994; Lamont, 1996; Clary, 1996).

In trials on various cytokines it was found that there<sup>air</sup> effect is strongly dependent on parameters such as 1) the site and manner of administration; 2) dosage and 3) duration of activity. Thus, for example, a systemic administration of recombinant IL-2 resulted less in the desired induction of an anti-tumoral immune response and more in serious toxic side effects and even therapy-induced deaths (Rosenstein et al., 1986; Rosenberg et al., 1989; Perez et al., 1991). The serious side effects resulted from the use of the cytokines whilst taking no account of their physiological mechanisms of activity. Cytokines are pleiotropic mediators which naturally serve to communicate between cells lying close together and operate over short distances. The release and place of activity of the cytokines are thus located close together, under physiological conditions (Pardoll, 1995). In order to obtain sufficiently high concentrations of the cytokine at the desired target site, when administering them systemically, high doses have to be given (Rosenberg et al., 1989) which lead to marked effects even at unwanted target locations.

The modern idea and the principle which a tumour vaccine sets out to achieve is to induce a systemic immune reaction directed against the tumour by means of a simultaneous, controlled delivery of appropriate tumour antigens and immunostimulatory cytokines, not by high-dose systemic administration of cytokines but rather by means of locally high doses of cytokines, acting over longer periods of time, at the site of vaccination (paracrine concept) (Pardoll, 1995; Jaffe et al., 1996).

However, the local administration of cytokines is technically problematic, particularly because the cytokines rapidly diffuse away (within minutes) into the surrounding tissues or into the blood system, coupled  
5 with an often extremely short half-life (inactivation) in biological fluids (Eppstein, 1982; Kedar et al., 1994; Koppenhagen, 1997).

In recent years it has been shown that a local release  
10 of cytokine, restricted to the vaccination site, and acting over a longer period of time can be achieved by injecting tumour cells which have been transfected with the gene coding for cytokine and have thus been made  
15 into cytokine producers (Fearon et al., 1990; Gansbacher et al., 1990; Rosenberg et al., 1992; Dranoff et al., 1993). It has been shown in animal models that vaccines based on cytokine gene-modified tumour cells are able to induce a systemic immune response directed specifically against the tumour, protecting the animals from a  
20 high-tumorigenic tumour challenge (Zatloukal et al., 1993; Zatloukal et al., 1995; Schmidt et al., 1995; Schweighoffer et al., 1996). In some cases, it was even possible to eliminate small tumours established before the vaccination (Clary et al., 1996; Clary et al.,  
25 1997). Although it is necessary to take account of the physiological paracrine mode of operation of the cytokines when adopting the approach using gene-modified tumour cells, the dosage of cytokine has proved to be another crucial parameter. Thus, it has been shown that  
30 in order to stimulate an immune response the cytokines have to be administered within a therapeutically effective dosage window; doses of cytokine which were too low were ineffective but so were excessively high doses (Zatloukal et al., 1995; Schmidt et al., 1995).  
35 On the other hand it is often difficult to achieve gene expression precisely within this effective dosage window

using the gene modification of tumour cells, especially primary tumour cells.

DE-A1 44 11 425 describes a cellular tumour vaccine  
5 which contains cytokines in delayed-release form. Specifically, it proposes IL-2 as the cytokine, taking no account of demands with regard to the dosage and release kinetics of the cytokine.

10 WO 94/21808 describes a tumour vaccine from autologous, cytokine gene-transfected cells, showing *inter alia* that the protective effect of cytokines (IL-2, IFN- $\gamma$  and GM-CSF) is dosage-dependent, and also demonstrating that  
15 the best protective effect is not necessarily achieved by the highest dose, but not defining an optimum dosage window.

The problem of the present invention was to provide an alternative tumour vaccine which is easy to produce,  
20 which makes it possible to release the immunostimulant cytokine in controlled manner at the vaccination site in the therapeutically effective dosage range over a fairly long period.

25 This problem is solved according to the invention with a tumour vaccine based on tumour antigens, which is characterised in that it contains, as active ingredient, in addition to a tumour antigen source, a release system with delayed release of the active substance for IFN- $\gamma$ ,  
30 the effective dosage of IFN- $\gamma$  being 50 ng to 5  $\mu$ g and the release interval being from half an hour to 8 days.

Preferably, the IFN- $\gamma$  dose is 100 ng to 2  $\mu$ g, particularly 100 ng to 1  $\mu$ g.

A release interval from half an hour up to 2 to 3 days has proved favourable; however, longer release intervals of up to 8 days have also demonstrated a favourable antitumour activity.

5

Preferably, at least about 75% of the effective IFN- $\gamma$  dose is released within a release period of from one hour to 3 days.

10 The release of IFN- $\gamma$  should start immediately if possible but at the latest one hour after administration of the vaccine. In any case, it is essential to the effectiveness of the tumour vaccine that the IFN- $\gamma$  and tumour antigen source should be available substantially  
15 simultaneously.

The release system with delayed release of active substance is hereinafter referred to as the "slow release system".

20

Fundamentally, within the scope of the present invention, all slow release systems which satisfy the requirements regarding dosage and release of IFN- $\gamma$  are suitable.

25

The slow release system preferably takes the form of liposomes.

Liposomes are synthetic lipid vesicles which consist of  
30 one or more concentric layers of lipid enclosing aqueous compartments. Water-soluble substances may be enclosed in the aqueous compartments whilst fat-soluble substances are incorporated in the lipid layers. Since these vesicles are suitable for a variety of uses owing  
35 to their structure, their biodegradability and low toxicity, they have increasingly been used in recent

years as carriers for all kinds of therapeutic active substances, including anti-tumour agents.

Liposomes are divided into two main categories. The first category includes the uni- or multilamellar "conventional" liposomes. Because they are rapidly absorbed by reticuloendothelial system these liposomes have a relatively short half-life.

10 In order to reduce non-specific interactions with cells of the reticuloendothelial system when the liposomes are used *in vivo* and in order to prevent excessively rapid breakdown, the liposomes are modified according to one embodiment of the invention. Preferably, the liposomes  
15 are modified with covalently bound polyethyleneglycol (PEG) ("PEGylated"; Mori et al., 1991; Chonn et al., 1992; Woodle et al., 1994). The quantity of PEG used is between 2 and 10% of PEG-coupled lipid in the liposome (m/m), the molecular weight of PEG is preferably between  
20 750 and 5000 D (Klibanov et al., 1990; Blume et al., 1990; Mayhew et al., 1992; Papahadjopoulos et al., 1991; Senior et al., 1991; Mori et al., 1991; Yoshioka, 1991). The liposomes may also be modified with other groupings such as amphiphilic vinyl polymers in order to prolong  
25 their half-life *in vivo*.

The prior art provides a wide variety of liposomes and methods of producing them which are suitable within the scope of the present invention; by way of example,  
30 reference is hereby made to US Patents 4,485,045 and 4,544,545; Epstein et al., 1985; Hwang et al., 1980; EP 0 036 676; EP 0 052 322; EP 0 088 046; EP 0 102 324; EP 0 142 641; EP 0 143 949; DE 3,218,121; Eppstein, 1982; Bergers et al., 1993; Kedar et al., 1994; Herrmann  
35 and Stricker, 1995; Koppenhagen, 1997. The effect of incorporating IFN- $\gamma$  in liposomes on their biological

activity has been described *inter alia* by Mellors et al., 1989 and Saravolac et al., 1996.

As an alternative to liposomes for the Slow Release  
5 System, biodegradable polymer materials in the form of  
microspheres may be used to incorporate IFN- $\gamma$  (Maulding,  
1987; Golumbek et al., 1993; Johnson et al., 1996; Lee  
et al., 1997; Cleland, 1997; Cleland and Jones, 1996) or  
Minipellets (Fujiwara et al., 1990; Marumo et al.,  
10 1997); these may also be modified to extend the half-  
life.

The source of tumour antigen for the vaccines according  
to the invention may be any compositions containing  
15 tumour antigens which are suitable for triggering a  
specific immune response in the individual being  
treated.

According to one embodiment of the invention the tumour  
20 antigens are present in the form of tumour cells.

The tumour cells of the vaccine may be autologous or  
allogenic tumour cells.

25 In one embodiment of the invention the tumour cells of  
the vaccines are autologous. These are cells which have  
been taken from the patient to be treated, optionally  
inactivated *ex vivo*, mixed with the slow release system  
which releases IFN- $\gamma$  and then re-administered to the  
30 patient (methods of preparing autologous tumour vaccines  
are known in the art and described *inter alia* in  
WO 94/21808, to the contents of which reference is  
hereby made.).

35 In a preferred embodiment of the invention the tumour  
cells are allogenic, i.e. they do not come from the

patient who is being treated (Barth et al., 1994; Morton et al., 1992). The use of allogenic cells which are generally available in the form of established tumour cell lines is preferred particularly when economic  
5 considerations come into play; the production of individual vaccines for each individual patient is labour intensive and costly and moreover in certain patients problems arise in ex vivo cultivation of the tumour cells, with the result that tumour cells are not  
10 obtained in sufficiently large numbers to produce a vaccine. Preferably, a mixture of cells from a number of allogenic tumour cells lines is used as the tumour antigen source. Tumour vaccines from allogenic tumour cells are known in the art; such vaccines have been  
15 described *inter alia* by Adler et al., 1995 and Hayashi et al., 1993; Oratz et al., 1989; Morton et al., 1989; Bystryn et al., 1986.

Lysates of tumour cells such as those described by  
20 Mitchell et al., 1993 may also be used as the antigen source.

In one embodiment of the invention the tumour antigen source consists of tumour cells, particularly allogenic  
25 tumour cells, charged with peptides derived from tumour antigens. One tumour vaccine which can be used according to the invention as an antigen source in conjunction with the IFN- $\gamma$  Slow Release Formulation was described by Buschle et al., 1997; as an alternative to  
30 tumour cells it is also possible to use antigen-presenting cells, e.g. dendritic cells which are charged with the tumour antigen peptides as described in DE-A1 196 07 044 or by Buschle et al., 1997.

35 The identification and isolation of tumour antigens and tumour-associated antigens (TAs) or peptides derived



therefrom (e.g. described by Wölfel et al., 1994 a) and 1994 b); Carrel et al., 1993; Lehmann et al., 1989; Tibbets et al., 1993; or in the published International Applications WO 92/20356, WO 94/05304, WO 94/23031, 5 WO 95/00159), was the prerequisite for using tumour antigens as such as immunogens, as described by Anchini et al., 1996.

In one embodiment of the invention the tumour antigens 10 are present in the form of tumour antigens as such in order to trigger a cellular immune response such as is required to eliminate tumour cells carrying tumour antigen (Bakker et al., 1994; Cox et al., 1994). The tumour antigens may be present in the form of proteins 15 or in the form of tumour antigen-derived peptides.

One example of a cell-free tumour vaccine based on tumour antigens or peptides derived therefrom which is suitable as an antigen source within the scope of the 20 present invention was described by Schmidt et al., 1996 and in WO 97/30721; reference is hereby made to these disclosures. A summary of cancer vaccines based on peptides which are suitable as antigen sources for the purposes of the present invention is provided by Melief 25 et al., 1996.

The antigen source may optionally also be in the form of a slow release formulation.

30 A tumour vaccine based on tumour antigens, e.g. in the form of tumour cells, in conjunction with a "slow release" system in which IFN- $\gamma$  is incorporated has the advantage, over tumour vaccines from gene-modified tumour cells which express IFN- $\gamma$ , that the release of 35 cytokine is precisely controlled at the vaccination site and hence the cytokine is administered in an accurate

and reproducible dosage. Moreover, the labour and hence costs involved in the manufacture are substantially reduced.

- 5 The results of the trials carried out within the scope of the present invention show that immunising mice with vaccines consisting of irradiated tumour cells and muIFN- $\gamma$ -liposomes can induce a systemic immune response which protects the animals from tumour development.
- 10 This protective effect demonstrates clear dependency on the dose of IFN- $\gamma$ . The most effective dosage range for immunisation was found to be in the range from 100 ng to 1  $\mu$ g of muIFN- $\gamma$ .

- 15 It has been shown that the protective effect is dependent not only on the IFN- $\gamma$  dose but also on the delayed release of the cytokine.

- The delayed release of liposome-encapsulated muIFN- $\gamma$
- 20 with regard to the antitumour protective effect was just as efficient as muIFN- $\gamma$  expressed by genetically modified cells. Free muIFN- $\gamma$  mixed in with the irradiated cells gave much reduced protection or none at all.

- 25 Both the quantity of incorporated IFN- $\gamma$  and its release at the activity site are dependent on the size, shape, structure and chemical composition of the particular slow release system used.

- 30 If desired, the vaccine according to the invention may contain one or more cytokines in addition to IFN- $\gamma$ , e.g. interleukin-2 (IL-2), IL-4, IL-12, IFN- $\alpha$ , IFN- $\beta$ , IFN- $\omega$ , TNF- $\alpha$ . The cytokine which may additionally be contained
- 35 in the vaccine may be present in the same slow release system as IFN- $\gamma$  or in a different one. The cytokine may

also be made available at the site of administration by using as the source of tumour antigen tumour cells (preferably allogenic) which are transfected with the corresponding cytokine gene.

5

Tumour cells modified in this way and processes for preparing them are described *inter alia* in WO 95/09655, WO 95/31107, WO 93/10219, WO 93/07906 and WO 94/21808 and by Belli et al., 1997.

10

The vaccine may optionally also contain non-specific immunostimulant adjuvants such as LPS (lipopolysaccharide) or BCG (Bacillus Calmette Guerin).

15 The vaccines may conveniently be formulated as follows:

Generally, the ratio of antigen to IFN- $\gamma$  dosage which is suitable for an efficient antitumour effect is determined first of all. One method of doing this is to start with a suboptimal amount of antigen for achieving an antitumour response (in the case of an optimum antigen dosage of about  $10^8$  tumour cells it might be, for example, about  $10^7$  cells) by titration, and determining the quantity of IFN- $\gamma$  at which the antitumour effect increases.

25

Then the definitive amount of antigen is optimised.

In order to provide an effective dosage of antigen the formulation generally contains about  $10^5$  to  $10^9$ , preferably  $10^6$  to  $10^8$  cells, when tumour cells are used as the antigen source; if a cell-free antigen source is used the quantity of tumour antigens or peptides derived from them is such that an immune response is triggered which is roughly equivalent to the immune response triggered by the number of tumour cells mentioned above.

35

If desired, another step may be carried out to check whether the addition of another cytokine or another non-specific adjuvant will bring about an increase in the antitumoral effect.

In order to select a slow release system which meets the requirements regarding the dosage of IFN- $\gamma$  and the release kinetics, the following process is preferably used (the term "liposomes" is hereinafter used to indicate all preparations which permit a delayed release of protein, e.g. microspheres or minipellets):

In order to test the efficiency of charging the liposomes with IFN- $\gamma$ , IFN- $\gamma$  is incorporated in various liposome preparations, free IFN- $\gamma$  is separated off and the IFN- $\gamma$  content of the liposomes is determined, e.g. by ELISA, HPLC or using the protein measuring method according to Lowry (Lowry et al., 1951). For the biological effectiveness of the vaccines according to the invention the absolute degree of charging the liposomes with IFN- $\gamma$  is not critical, but the dosage available within a certain interval of time is essential. However, it has proved advantageous if the IFN- $\gamma$  concentration in the liposome preparation is at least about 10  $\mu\text{g/ml}$ , preferably more than 20  $\mu\text{g/ml}$ . (With a low absolute level of charging of the liposomes the desired immunomodulatory effect of IFN- $\gamma$  in the vaccine can be achieved by increasing the proportion of liposome preparation in the vaccine).

In a preferred embodiment, at least 90% of the IFN- $\gamma$  is enclosed in the liposomes, i.e. not more than 10% of the protein is adsorbed on the outside of the liposomes. One advantageous method of producing "alternative" liposomes of this kind consists in reducing or

preventing the non-specific electrostatic interaction which leads to the adsorption of IFN- $\gamma$ , by providing a washing step using a saline solution (e.g. NaCl). The concentration of the ions must be sufficient to compete  
5 with the protein for binding to the liposomes.

In a subsequent step the release kinetics of the liposomes are determined.

- 10 In the case of liposomes the incorporation or release kinetics of the cytokines is dependent on the charge, the hydrophilic/hydrophobic nature of the cytokine, on the one hand, and on the chemical and physico-chemical characteristics of the liposomes, on the other hand.
- 15 The most important characteristics of the liposomes which determine their release kinetics are their size, the number of lipid layers (uni-/multilamellar), the charge and fluidity of the lipid layers. These are in turn determined by the chemical composition of the lipid  
20 layers (Eppstein, 1982; Koppenhagen, 1997).

The principle of suitable tests for determining the release kinetics is based on incubating IFN- $\gamma$ -charged liposomes in a physiological buffer system which  
25 contains serum in order to simulate conditions *in vivo* and determining the IFN- $\gamma$  concentration in the supernatant at specific time intervals, e.g. by ELISA. The specialist literature relating to Slow Release Systems describes specific tests for determining the  
30 release kinetics of the therapeutic agent.

Preferably, the release kinetics are determined *in vivo*, in addition to the tests *in vitro*. Analogously to the form of administration finally adopted for therapeutic  
35 use (site of vaccination, route of administration), experimental animals are given the corresponding

injection volume which, for reasons of detectability, usually contains a larger amount of IFN- $\gamma$  than the active dose of vaccine envisaged. Then blood samples are taken from the experimental animals at specified  
5 time intervals and the IFN- $\gamma$  content is determined, e.g. using ELISA.

Alternatively, the *in vivo* release kinetics can be determined by administering a liposome preparation in  
10 which the liposomes and the IFN- $\gamma$  contained therein have different radiolabelling. After the injection, samples are taken from the vaccination site at specified time intervals and the residual radioactivity is determined. The absolute values indicate any liposome/IFN- $\gamma$  still  
15 present at the injection site; a proportional decrease in the two values leads one to conclude that the IFN- $\gamma$  is still encapsulated in the liposomes.

The effective combination of tumour antigens/IFN- $\gamma$   
20 contained in the tumour vaccine according to the invention is present in such a way as to trigger a cytotoxic T-cell response and/or a humoral immune response which eliminates the tumour cells or, in the case of preventive use, ensures protection from tumour  
25 formation.

There are tests available to the skilled person for determining the extent of the immune response and for deciding on the optimum dosage of tumour antigen/IFN- $\gamma$   
30 on the basis of the test results.

The triggering of a cellular immune response can be confirmed by detecting antigen-specific CTLs (Coligan et al., 1991). Further evidence of the presence of a  
35 cellular immune response is provided if, in the absence of T-cells in an experimental animal (which is achieved

by treating the animal with antibodies which deplete CD4- or CD8-cells) no immune response takes place (Coligan et al., 1991).

- 5 A cellular immune response can also be indicated by detecting a "delayed-type hypersensitivity" (DTH) reaction in immunised animals. To do this, peptides are injected into the soles of the feet of mice and the swelling at the injection site is measured (Grohman et al., 1995; Puccetti et al., 1994). In order to measure the DTH reaction in the patient, antigens are injected intradermally and the reddening or swelling at the injection site are measured.
- 10
- 15 The induction of a humoral immune response by antigens or peptides derived therefrom which are foreign antigens to the body or antigens which are expressed in low concentrations by the body to be treated, can be determined by detecting specific antibodies in the serum. One suitable method of determining the antibody titre in the serum is enzyme linked immunoassay (ELISA). The specific antibodies are detected by a reaction with a dye after binding to the peptide used for immunisation. An alternative method is the Western blot. In this, specific serum antibodies bind to the peptide immobilised on a membrane. Bound antibodies are finally detected by reaction with a dye (Coligan et al., 1991).
- 20
- 25
- 30 In the next step, the immunomodulatory effect of the vaccines is measured in an animal trial. Established tumour models in which irradiated tumour cells induce at least a small immune response, or tumour models in which there are known tumour antigen peptide sequences recognised by immune cells, may be used for this purpose, *inter alia*. The vaccine is administered in
- 35

varying ratios of tumour antigen source to IFN- $\gamma$  slow release system. The protection from tumour growth is a measurement of the effectiveness of the tumour vaccines.

- 5 The injection volume is generally 50  $\mu$ l to 2 ml.

The vaccine according to the invention is generally in the form of a suspension in a pharmaceutically acceptable carrier, preferably an aqueous carrier.

- 10 Aqueous carriers which may be used include for example water, buffered water, saline solution (0.4%), glycine solution (0.3%), hyaluronic acid and other known carriers. The composition may also contain  
15 pharmaceutically acceptable adjuvants such as buffer substances and inorganic salts in order to achieve normal osmotic pressure and/or effective lyophilisation. Examples of such additives include sodium- and potassium salts, e.g. chlorides and phosphates, saccharose, glucose, hydrolysed protein, dextran,  
20 polyvinylpyrrolidone or polyethyleneglycol. The compositions may be sterilised using conventional techniques, e.g. sterile filtration. The composition may be packaged directly in this form or freeze-dried and mixed with a sterile solution before use.

- 25 If desired, the tumour vaccine according to the invention may be packaged in the form of two separate formulations (tumour antigen source and IFN- $\gamma$  slow release formulation) which are combined before being  
30 administered.

- The tumour vaccine according to the invention may be administered prophylactically or therapeutically. It is preferably administered by intradermal, subcutaneous or  
35 intramuscular route.



### Summary of the Figures

- 5 Fig. 1: Determining the release kinetics of muIFN- $\gamma$  from liposomes *in vitro*
- Fig. 2: Immunisation of mice with a vaccine from tumour cells and muIFN- $\gamma$  (1<sup>st</sup> experiment)
- 10 Fig. 3: Immunisation of mice with a vaccine from tumour cells and muIFN- $\gamma$  (2<sup>nd</sup> experiment)
- 15 Fig. 4: Determining the cytotoxic activity of T-lymphocytes after immunisation with a vaccine from tumour cells and muIFN- $\gamma$

#### Example 1

##### 20 Incorporation of human IFN- $\gamma$ in liposomes

Optimising the chemical composition and method of preparing the liposomes

25 Since the association of IFN- $\gamma$  and liposomes is primarily charge-dependent, preliminary trials were carried out with human IFN- $\gamma$ , in which the charge of the liposomes was varied. It was found that as the negative charge of the liposomes increased the efficiency of the encapsulation or association of IFN- $\gamma$  improved.

30 For these trials, the molar ratio of the negative and neutral phospholipids (egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG)) was changed.

It was found that the association efficiency was only 20 % with a molar ratio of 1:0, whereas with a molar  
35 ratio of 1:4, 4:1 or 9:1 it rose to more than 95 %.

It was also established that the presence of salt in the hydration medium during the encapsulation reduces the efficiency of the incorporation of IFN- $\gamma$  in liposomes; e.g. the rate of incorporation fell from 90 % to 35 % at a ratio of PC:PG=9:1 after the addition of 0.9 % NaCl. Consequently, instead of physiological saline solution a 5 % glucose solution was added in order to obtain an isotonic solution (this is necessary for administration).

10

For subcutaneous (s.c.) administration, large unsized liposomes (multilamellar vesicles) were used, because the formation of an s.c. deposit is necessary for the optimum immunostimulant activity when the vaccination is used for prophylactic or therapeutic purposes and only large liposomes remain at the injection site for a fairly long time.

The additional incorporation of cholesterol (PC:PG:cholesterol=5:1:4) in the lipid bilayers, which makes the liposomes more rigid, had no effect on either the rate of incorporation or the release kinetics of IFN- $\gamma$ .

25 Stability tests on liposomes (PC:PG:cholesterol=5:1:4) at 4°C showed no change in stability.

The liposomes containing IFN- $\gamma$  proved to be stable for at least one month at 4°C. After 30 days an IFN- $\gamma$ -activity of more than >80 %, determined by HPLC, was demonstrated. The 20 % reduction in activity can clearly be attributed to a breakdown of IFN- $\gamma$ ; the protein measurement according to Lowry et al., 1951, showed that no appreciable amounts of protein were released from the liposomes (the storage of free IFN- $\gamma$  at 4°C also shows a 20 % loss of activity).

## Example 2

Induction of a systemic immune response to melanoma  
5 cells by immunisation with vaccines consisting of a  
mixture of irradiated tumour cells and muIFN- $\gamma$ -liposomes

### a) Preparation of muIFN- $\gamma$ -liposomes:

10 Recombinant murine IFN- $\gamma$  was diluted in 10 mM succinate  
buffer (10 mM sodium succinate, pH=5, 5% glucose) to a  
concentration of 100  $\mu$ g/ml.

The liposomes were prepared by hydration of a lipid film  
15 as follows: Egg phosphatidylcholine (EPC, Lipoid GmbH,  
Ludwigshafen) and egg phosphatidylglycerol (EPG, Lipoid  
GmbH, Ludwigshafen) (in a molar ratio of 9:1) were  
dissolved in 5 parts by volume of ethanol/methanol  
(=solvent). (100  $\mu$ mol of lipid correspond to 75 mg;  
20 0.9 x 40 x 0.75 mg/ml of EPC and 0.1 x 40 x 0.75 mg/ml  
of EPG were used accordingly.) The organic solvent was  
evaporated *in vacuo* in a rotary flask. The thin lipid  
film thus obtained was dissolved with the IFN- $\gamma$  solution  
by swirling the flask with glass beads. Using this  
25 method, mainly multilamellar liposomes (MVL) were  
obtained in which the IFN- $\gamma$  is enclosed or onto which  
some of the IFN- $\gamma$  may optionally be adsorbed. Any  
unincorporated or unadsorbed IFN- $\gamma$  was separated off by  
ultracentrifugation at 250 000 x g for 60 min in 10 mM  
30 succinate buffer, pH=5, 10% saccharose. The liposomes  
contained about 86  $\mu$ g of IFN- $\gamma$ /ml and 36  $\mu$ mol/ml of  
phospholipids. They were stored at 4°C.

b) Determining the release kinetics of the muIFN- $\gamma$  from  
35 the liposomes *in vitro*

In order to determine the release kinetics of IFN- $\gamma$  from the liposomes, liposomes containing IFN- $\gamma$  were incubated in PBS/10%FCS buffer (about 0.5 - 1 ml liposomes per ml of buffer) at 37°C, which simulates an *in vivo* environment, for the time spans given in Fig.1. After incubation the samples were mixed with saccharose solution and centrifuged in order to separate the liposomes and the released protein. The lower phase was separated from the liposomes and frozen at -20°C until the IFN- $\gamma$  content was determined. The content of IFN- $\gamma$  was determined using an ELISA (BIO Source).

It was found that there was a delayed release of the cytokine from the liposomes. The majority of the cytokine is released within the 1<sup>st</sup> day. However, the release continues for several days and is still detectable after 8 days (Fig.1).

#### c) Mouse melanoma cells

The mouse melanoma cell line B16F10 (Fidler et al., 1975) comes from the NIH DCTDC Tumor Repository. The cells were cultivated in T175 culture bottles in DMEM, 10% FCS, 2 mM glutamine.

#### d) Preparation of the vaccines

B16F10 melanoma cells ( $1-2 \times 10^7$  cells per T175 culture bottle) were irradiated with  $\gamma$ -rays at a dose of 50 Gy in order to suppress further multiplication of the cells. 2-6 h after irradiation the cells were trypsinised with a trypsin/EDTA solution, washed in 3 washing steps with culture medium, PBS and Ringer's solution and adjusted to a concentration of  $4 \times 10^6$  cells per ml (in Ringer's solution).

The cell suspension was mixed in equal parts by volume with liposome suspensions containing muIFN- $\gamma$  in various concentrations (100  $\mu\text{g/ml}$ , 20  $\mu\text{g/ml}$ , 4  $\mu\text{g/ml}$ , 0.8  $\mu\text{g/ml}$  and 0  $\mu\text{g/ml}$ ). The finished vaccines thus contained a  
5 tumour cell concentration of  $2 \times 10^6$  cells per ml and concentrations of liposomally encapsulated muIFN- $\gamma$  corresponding to: 50  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$ , 2  $\mu\text{g/ml}$ , 0.4  $\mu\text{g/ml}$  and 0  $\mu\text{g/ml}$ .

10 e) Immunisation of the mice

1st experiment:

Syngenic C57Bl/6 mice (female, 8 weeks old) were  
15 immunised by subcutaneous injection of the vaccines into the rear right-hand flank. The fur was abraded on both rear flanks for this purpose. The injection volume was 100  $\mu\text{l}$  per mouse. Thus  $2 \times 10^5$  B16 cells and correspondingly 5  $\mu\text{g}$ , 1  $\mu\text{g}$ , 200 ng or 40 ng of  
20 liposomally encapsulated muIFN- $\gamma$  were administered in each immunisation per mouse. 8 mice were immunised per dosage group.

Parallel thereto, groups of 8 mice were immunised with  
25 the following control vaccines :

- irradiated B16 cells
  - irradiated B16 cells + empty(=placebo) liposomes
  - irradiated B16-cells + free mu IFN- $\gamma$  5 $\mu\text{g/ml}$
  - mu IFN- $\gamma$  genetically modified, irradiated B16-cells
- 30 (the transfection was carried out with the adenovirus-aided gene transfer system based on receptor-mediated endocytosis, as described in WO 94/21808 and by Zatloukal et al., 1995; the release of mu IFN- $\gamma$  was 120 ng per 24 h).

One week after the first immunisation the mice were given a booster immunisation with the same vaccines as had been used for the first immunisation.

- 5 After another week the animals were exposed to the highly tumorigenic dose of  $1 \times 10^5$  B16 cells which was administered at a site remote from the immunisation site (on the opposite side). In addition, a group (8 animals) of non-immunised mice were exposed to the tumorigenic  
10 cells in the same way.

- Eight weeks after the implanting of the tumour cells all (8/8) the unimmunised mice had developed tumours. By contrast, 4 out of 8 and 3 out of 8 animals of the  
15 groups which had been immunised with irradiated cells and muIFN- $\gamma$ -liposomes, containing 200 ng or 1  $\mu$ g of muIFN- $\gamma$ , were protected. A similar protective effect (3/8 tumour-free animals) was found for animals which had been immunised with muIFN- $\gamma$ -genetically modified cells.  
20 A merely marginal (insignificant) effect (1 out of 8 protected animals) was found for irradiated cells + liposomes containing 40 ng of mu IFN- $\gamma$ -, irradiated cells + free mu IFN- $\gamma$  (5  $\mu$ g) and for irradiated cells + empty liposomes. Irradiated cells on their own or  
25 irradiated cells + liposomes containing 5  $\mu$ g of mu IFN- $\gamma$  had no protective effect. The development of the tumours in the animals is summarised in Table I and Fig. 2.

2nd experiment:

- 30 Syngenic C57Bl/6 mice (female, 9 weeks old) were immunised by subcutaneous injection of the vaccines into the right-hand hind flank. For this purpose the fur had been abraded on both hind flanks. The injection volume  
35 was 100  $\mu$ l per mouse.  $2 \times 10^5$  B16 cells and liposomally encapsulated muIFN- $\gamma$  in doses of 4  $\mu$ g, 1  $\mu$ g, 200 ng or

40 ng were administered to each mouse per immunisation.  
8 mice were immunised per dosage group.

Parallel thereto, groups of 8 mice were immunised with  
5 the following control vaccines :

- irradiated B16 cells
- irradiated B16-cells + empty (=placebo) liposomes
- irradiated B16-cells + free muIFN- $\gamma$  4  $\mu$ g/ml
- irradiated B16-cells + free muIFN- $\gamma$  1  $\mu$ g/ml
- 10 - irradiated B16-cells + free muIFN- $\gamma$  200 ng/ml
- muIFN-g-genetically modified, irradiated B16-cells  
(the release of muIFN- $\gamma$  was 200 ng per 24 h).

One week after the first immunisation the mice were  
15 given a booster immunisation with the same vaccines as  
had been used for the first immunisation.

After another week the animals were exposed to the  
highly tumorigenic dose of  $1 \times 10^5$  B16 cells which was  
20 administered at a site remote from the immunisation site  
(on the opposite side). In addition, a group (8 animals)  
of non-immunised mice were exposed to the tumorigenic  
cells in the same way.

25 Eight weeks after the implanting of the tumour cells all  
(8/8) of the unimmunised mice had developed tumours. By  
contrast, 4 out of 8 and 3 out of 8 animals of the  
groups which had been immunised with irradiated cells  
and muIFN- $\gamma$ -liposomes, containing 1  $\mu$ g or 200 ng of  
30 muIFN- $\gamma$ , were protected. A similar protective effect (4/8  
tumour-free animals) was found for animals which had  
been immunised with muIFN- $\gamma$ -genetically modified cells.  
A slight protective effect (1 out of 8 animals  
protected) was found for irradiated cells + liposomes  
35 containing 40 ng of muIFN- $\gamma$  and for irradiated cells +  
free muIFN- $\gamma$  (1  $\mu$ g). Irradiated cells or irradiated cells

+ muIFN- $\gamma$  liposomes containing 4  $\mu$ g of muIFN- $\gamma$  as well as vaccines consisting of irradiated cells + free muIFN- $\gamma$  (4  $\mu$ g or 200 ng) had no protective effect. The development of the tumours in the animals is summarised in Table II and Fig.3.

### Example 3

Determining the cytotoxic activity of T-lymphocytes of vaccinated animals

Syngenic C57Bl/6 mice (female, 9 weeks old) were immunised by subcutaneous injection of the vaccines into the right-hand hind flank. For this purpose the fur had been abraded on both hind flanks. The injection volume was 100  $\mu$ l per mouse.  $2 \times 10^5$  B16 cells and liposomally encapsulated muIFN- $\gamma$  in doses of 3.8  $\mu$ g (=high dose) and 300 ng (optimum dose) were administered to each mouse per immunisation. Parallel thereto a group was immunised only with irradiated B16 cells and another group was injected only with buffer (control group). 4 mice were immunised per group.

One week after the first immunisation the mice were given a booster immunisation with the same vaccines as had been used for the first immunisation.

After 13 days the animals were killed and their spleens were removed. The splenocytes were isolated, pooled within each group and restimulated for 5 days *in vitro* with irradiated B16 cells. On the 6<sup>th</sup> day the cytotoxic activity (CTL activity) of the splenocytes (effector cells) against B16 cells (target cells) was measured using a Europium release assay (Blomberg et al., 1993). The CTL activity (expressed as % specific lysis (Zatloukal et al., 1993)) was determined for various



effector:target ratios (100:1, 50:1, 25:1) and is shown in Fig.4. Splenocytes from mice which had been immunised with irradiated B16 cells and muIFN- $\gamma$ -liposomes in the optimum dosage range (300 ng of muIFN- $\gamma$ ) exhibited a clear cytotoxic activity against B16 cells (10 % specific lysis) compared with a very slight background activity in control animals or animals which had been immunised only with irradiated B16 cells. The increase in the muIFN- $\gamma$  dose to 3.8  $\mu$ g (already a high dose) led to a reduction in the CTL activity compared with the optimum dose.

#### Example 4

Determining the release kinetics of muIFN- $\gamma$  at the vaccination site

Once it was found that muIFN- $\gamma$  liposomes are an efficient adjuvant in a cellular tumour vaccine and free muIFN- $\gamma$  provided no protection against a lethal tumour challenge, it was assumed that the encapsulation in liposomes results in a prolonged presence of the cytokine at the activity site. The release of muIFN- $\gamma$  liposomes was therefore investigated in order to show that the liposomes stay at the injection site, the site of antigen presentation. muIFN- $\gamma$  liposomes were prepared as described in Example 2a, except that the final concentration of muIFN- $\gamma$  was 5  $\mu$ g/ml.

In order to be able to monitor the persistence of muIFN- $\gamma$  at the injection site,  $^{125}$ I-labelled muIFN- $\gamma$  was used (the specific activity corresponded to that of the  $^{125}$ I-labelled huIFN- $\gamma$  described in the following Example, likewise the mixing ratio to non-labelled muIFN- $\gamma$ ). In order to monitor the persistence of the liposomes at the injection site, in an alternative approach, when

preparing the lipid film,  $1\alpha,2\alpha$  (n)-[ $^3\text{H}$ ]-cholesteryl-  
ether, specific activity: 46 mCi (1.71 Tbq)/mMol  
(Amersham) was used (10  $\mu\text{l}$ ) in addition to PC and PG.  
As described in Example 2e, a single dose of 100  $\mu\text{l}$  of  
5 liposomes ([ $^{125}\text{I}$ ]-muIFN- $\gamma$  liposomes or [ $^3\text{H}$ ] liposomes) or  
free [ $^{125}\text{I}$ ]-muIFN- $\gamma$  (5  $\mu\text{g}/\text{ml}$ ) was injected into the mice  
subcutaneously, into the right-hand hind flank. Unlike  
Example 2e, no cells were added. After various lengths  
of time (see Fig. 5) the mice were killed. The injection  
10 site was divided into three parts. Each piece was placed  
in a glass scintillation test tube and 3 ml of Soluene-  
350 (Packard) were added in order to dissolve the  
samples for three days at  $40^\circ\text{C}$ . 500  $\mu\text{l}$  of the dissolved  
sample were pipetted into a clean glass test tube and  
15 250  $\mu\text{l}$  of  $\text{H}_2\text{O}_2$  were added, the test tubes being sealed  
once foaming had stopped. The samples were bleached for  
24 h, then the addition of 250  $\mu\text{l}$  of  $\text{H}_2\text{O}_2$  was repeated  
twice until the samples were colourless. Finally,  
Hionic-Fluor (Packard) was added as the scintillation  
20 fluid and the samples were mixed in a vortex. The [ $^{125}\text{I}$ ]  
radioactivity was measured after 24 h in a Packard  
multi-priar-2 $\gamma$ -Counter and the [ $^3\text{H}$ ] radioactivity was  
measured in a Philips PW 4700 Liquid Scintillation  
Counter. Fig. 5 shows the percentage of the dose  
25 remaining at the injection site (ID), based on the total  
dose injected. It was found that the liposomally  
encapsulated muIFN- $\gamma$  stays longer at the injection site  
than the free muIFN- $\gamma$ .

### 30 Example 5

Determining the release kinetics of huIFN- $\gamma$  at the  
vaccination site

#### 35 a) Preparation of "conventional" huIFN- $\gamma$ liposomes

The liposomes (40  $\mu\text{Mol/ml}$ ) were prepared by the film method, substantially as described in Example 1 or Example 2, the PC and PG being mixed in a molar ratio of 9:1. For the preparation of [ $^{125}\text{I}$ ]-huIFN- $\gamma$  liposomes,  
5 3.5 ml (50  $\mu\text{g/ml}$ ) of huIFN- $\gamma$  were added to 5 % glucose (10 mM Na-succinate buffer, pH 5.0) plus 79  $\mu\text{l}$  of [ $^{125}\text{I}$ ]-huIFN- $\gamma$  (specific activity: 13.8  $\mu\text{Ci/ml}$ ; 10 kBq/mouse). The films were hydrated by manually shaking the flasks.

10 b) Preparation of "alternative" huIFN- $\gamma$  liposomes

Since in preliminary trials the "conventional" liposome formulation had shown the release of a high proportion of [ $^{125}\text{I}$ ]-huIFN- $\gamma$  during the first hours, which can  
15 presumably be put down to the fact that this proportion immediately detaches itself from the liposomes and disappears from the vaccination site, "alternative" liposomes were prepared which contain less protein (< 10 %) adsorbed on the outer membrane.

20 For this purpose, lipid films consisting of PC and PG in a molar ratio of 9:1 were hydrated with a very small volume of huIFN- $\gamma$  in 10 % saccharose (10 mM Na-succinate buffer, pH 5.0), e.g. 0.5  $\mu\text{l}$ ; 500  $\mu\text{g/ml}$ . A highly  
25 viscous, gelatinous mass of highly concentrated liposomes was obtained. After 30 min this was diluted to the normal injectable concentration (40 mM of total lipid; 5  $\mu\text{g/ml}$  of huIFN- $\gamma$ ) and washed in 0.9 % NaCl in  
30 10 mM Na-succinate, pH 5. As the salt screens the negative charges of the lipid membrane, it prevents the electrostatic interaction with the positively charged huIFN- $\gamma$ . The non-encapsulated huIFN- $\gamma$  was removed by ultracentrifugation. After two washes the liposomes were taken up in 10 % saccharose buffer. The incorporation of  
35 [ $^{125}\text{I}$ ]-huIFN- $\gamma$  was carried out as described in a).

In order to determine the proportion of liposome-bound protein adsorbed on the outside of the liposomes, the liposomes are incubated in a trypsin solution: 20 ml (1 mg/ml) of trypsin in phosphate-buffered saline, pH 7.4, were added to 100 ml (100 mg/ml) of the liposomal dispersion (100 mg/ml of trypsin was sufficient to break down 250 mg/ml of protein). After 1 h incubation at 37°C, the enzymatic reaction was broken off during the extraction of the samples; the quantity of encapsulated huIFN- $\gamma$  was determined by HPLC; it was >90 %.

c) Preparation of solution containing free huIFN- $\gamma$

The "free" [ $^{125}$ I]-huIFN- $\gamma$  solution (10 kBq/mouse) was prepared by adding 52  $\mu$ l of [ $^{125}$ I]-huIFN- $\gamma$  stock solution (120  $\mu$  Ci/ml) to 2.3 ml (50  $\mu$ g/ml) of huIFN- $\gamma$  in 5 % glucose (10 mM Na-succinate buffer, pH 5.0).

d) *in vivo* test

The two liposome preparations and the solution containing free huIFN- $\gamma$  were injected into the mice as described in the previous Examples and the injection dose remaining was measured as described in Example 4. The results of the measurements are shown in Fig. 6, giving the percentage of the dose remaining at the injection site (ID), based on the total dose injected. It was found that the liposomally encapsulated huIFN- $\gamma$  persists longer at the injection site than the free huIFN- $\gamma$ , while the „alternative“ liposomes exhibited even more favourable properties in terms of sustained release.

**Table I**

	<b>Tumour-free mice (%) after the stated number of weeks</b>								
<b>vaccination</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>
buffer	62.5	0	0	0	0	0	0	0	0
B16	87.5	12.5	0	0	0	0	0	0	0
liposomes+B16	100	25	12.5	12.5	12.5	12.5	12.5	12.5	12.5
IFN liposomes (5 µg)+B16	100	12.5	0	0	0	0	0	0	0
IFN liposomes (1 µg)+B16	100	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5
IFN liposomes (200 ng)+B16	100	62.5	50	50	50	50	50	50	50
IFN liposomes (40 ng)+B16	100	37.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
free IFN + B16	100	37.5	25	12.5	12.5	12.5	12.5	12.5	12.5
free IFN + liposomes + B16	100	37.5	25	25	25	25	25	25	25
IFN/B16	100	62.5	50	37.5	37.5	37.5	37.5	37.5	37.5
	<b>Number of tumour-free mice per number of injected mice after the stated number of weeks</b>								
<b>vaccination</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>
buffer	(5/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
B16	(7/8)	(1/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
liposomes+B16	(8/8)	(2/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)
IFN liposomes (5 µg)+B16	(8/8)	(1/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
IFN liposomes (1 µg)+B16	(8/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)
IFN liposomes (200 ng)+B16	(8/8)	(5/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)
IFN liposomes (40 ng)+B16	(8/8)	(3/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)
free IFN + B16	(8/8)	(3/8)	(2/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)
free IFN + liposomes + B16	(8/8)	(3/8)	(2/8)	(2/8)	(2/8)	(2/8)	(2/8)	(2/8)	(2/8)
IFN/B16	(8/8)	(5/8)	(4/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)

B16 = B16F10 melanoma cells irradiated with a dose of 50 Gy  
 IFN/B16 = B16F10 melanoma cells transfected with the gene for IFN-gamma and irradiated with 50 Gy

**Table II**

	<b>Tumour-free mice (%) after the stated number of weeks</b>								
<b>Vaccination</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>
Buffer	37.5	0	0	0	0	0	0	0	0
B16	87.5	37.5	0	0	0	0	0	0	0
Liposomes + B16	87.5	25	0	0	0	0	0	0	0
IFN liposomes (4 µg) + B16	100	12.5	0	0	0	0	0	0	0
IFN liposomes (1 µg) + B16	100	62.5	62.5	50	50	50	50	50	50
IFN Liposome (200 ng) + B16	100	62.5	62.5	37.5	37.5	37.5	37.5	37.5	37.5
IFN liposomes (40 ng) + B16	100	50	50	37.5	25	25	12.5	12.5	12.5
free IFN (4 µg) + B16	100	25	25	0	0	0	0	0	0
free IFN (1 µg) + B16	100	37.5	25	12.5	12.5	12.5	12.5	12.5	12.5
free IFN (200) + B16	100	0	0	0	0	0	0	0	0
IFN/B16 (200 ng/24h)	100	62.5	62.5	62.5	62.5	62.5	50	50	50
	<b>Number of tumour-free mice per number of injected mice after the stated number of weeks</b>								
<b>Vaccination</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>
buffer	(3/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
B16	(7/8)	(3/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
liposomes + B16	(7/8)	(2/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
IFN liposomes (4 µg) + B16	(8/8)	(1/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
IFN liposomes (1 µg) + B16	(8/8)	(5/8)	(5/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)
IFN liposomes (200 ng) + B16	(8/8)	(5/8)	(5/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)
IFN liposomes (40 ng) + B16	(8/8)	(4/8)	(4/8)	(3/8)	(2/8)	(2/8)	(1/8)	(1/8)	(1/8)
free IFN (4 µg) + B16	(8/8)	(2/8)	(2/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
free IFN (1 µg) + B16	(8/8)	(3/8)	(2/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)
free IFN (200) + B16	(8/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
IFN/B16 (200 ng/24h)	(8/8)	(5/8)	(5/8)	(5/8)	(5/8)	(5/8)	(4/8)	(4/8)	(4/8)

B16 = B16F10 melanoma cells irradiated with a dose of 50 Gy

IFN/B16 = B16F10 melanoma cells transfected with the gene for IFN-gamma and irradiated with 50 Gy

# Literature

- Adler, A., et al., (1995), Cancer Biother 10(3): 211-224
- 5 Anchini, A., Mortarini, R., Maccalli, C., Squarcina, P., Fleischbauer, K., Mascheroni, L., and Parmiani, G. (1996), J. Immunol. 156: 208-217
- Bakker, A.B.H., et al., (1994), J. Exp. Med. 179: 1005-1009
- 10 Barth, A., Hoon, D., Foshag, L., Nizze, J., Famatiga, E., Okun, E. and Morton, D. (1994), Cancer Res. 54: 3342-3345
- Belli, F., et al., (1997), Cancer Immunol Immunother 44(4): 197-203
- 15 Berd, D., Maguire HC., McCue P. (1990), J. Clin. Oncol. 8: 1858-1863
- Bergers, J.J., Den Otter, W., Dullens, H.F.J., Kerkvliet, C.T.M., and Crommelin, D.J.A. (1993), Pharmaceut. Research 10, 12: 1715-1721
- 20 Blomberg, K. and Ulfstedt, A.C., (1993), J. Immunol. Methods 160: 27-34
- Blume et al, (1990), Biochim Biophys. Acta 1029: 91-7
- Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., DePlaen, E., Lethe, B., Coolie, P., Boon, T. (1993), J. Exp. Med. 178: 489-495
- 25 Buschle, M., et al., (1997), Proc. Nat. Acad. Sci. USA 94(7): 3256-3261
- Bystryn, J.C., et al., (1986), J Biol Response Mod 5(3): 211-224
- 30 Carrel, S. and Johnson, J.P., 1993, Current Opinion in Oncology 5, 383-389
- Chonn, A.; Semple, S. C.; Cullis, P. R., (1992), J Biol Chem 267: 18759-65
- Clary, B.M., Coveney, E., Blazer, D.G., Philip, R., and 35 Lysterly, H.K. (1996), Surgery 120: 174-181
- Clary, B.M., Coveney, Philip, R., Blazer, D.G., Morse, M., Gilboa, E., and Lysterly, H.K. (1997), Cancer Gene Therapy 4: 97-104
- Cleland, J.L. and Jones, A.J., (1996), Pharm. Res. 40 13(10): 1464-1475
- Cleland, J.L., (1997), Pharm. Biotechnol. 10:1-43
- Coligan, J.E., et al., (1991), Current Prot. in Immunol., Kapitel 3, Wiley, New York
- Coulie, P., Brichard, V., Van Pel, A., Wolfel, T., 45 Schneider, J., Traversari, C., Mattei, S., De Plaen, E., Lurquin, C., Szikora, J-p., Renauld, J-c., Boon, T. (1994), J. Exp. Med. 180: 35-42

- Cox, A.L., et al., (1994), Science 264: 716-719
- Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P.,  
Levitsky, H., Brose, K., Jackson, V., Hamada, H.,  
Pardoll, D. and Mulligan, R.C. (1993), Proc. Natl.  
5 Acad. Sci. USA 90: 3539-43
- Eppstein, D.A. (1982), J. Interferon Research 2, 1:  
117-125
- Epstein et al., (1985), Proc. Natl. Acad. Sci. (USA) 82:  
3688-3692
- 10 Fearon, E.R., Pardoll, D.M., Itaya, T., Golumbek, P.,  
Levitsky, H., Simons, J.W., Karasuyama, H.,  
Vogelstein, B. and Frost, P. (1990), Cell 60:  
397-403
- Ferrantini, M., Giovarelli, M., Modesti, A., Musiani,  
15 P., Modica, A., Venditti, M., Peretti, E., Lollini,  
P.-L., Nanni, P., Forni, G., and Belardelli, F.  
(1994), J. Immunol. 153: 4604-4615
- Fidler et al., (1975), Cancer Res. 35: 218-234
- Fujiwara, T., Sakagami, K., Matsuoka, J., Shiozaki, S.,  
20 Uchida, S., Fujioka, K., Takada, Y., Onoda, T., and  
Orita, K. (1990), Cancer Research 50: 7003-7007
- Gansbacher, B., Zier, K., Daniels, B., Cronin, K.,  
Bannerji, R., and Gilboa, E. (1990), J. Exp. Med.  
172: 1217-1224
- 25 Gaughran, B., Van den Eynde, B., van der Bruggen, P.,  
Romero, P., Gaforio, J.J., De Plaen, E., Lethe, B.,  
Brasseur, F., Boon, T. (1994), J. Exp. Med. 179:  
921-930
- Golumbek, P.T., Azhari, R., Jaffee, E.M., Levitsky,  
30 H.I., Lazenby, A., Leong, K., and Pardoll, D.M.  
(1993), Cancer Research 53(24): 5841-5844
- Grohmann, U. et al., (1995), Eur. J. Immunol. 25,  
2797-2802
- Hayashi, Y., et al., (1993), Cancer 72(3): 750-759
- 35 Herrmann, J. and Stricker, H., (1995), Eur. J. Pharm.  
Biopharm. 41 (6): 361-368
- Hwang et al., (1980), Proc. Natl. Acad. Sci. (USA) 77:  
4030-4034
- Jaffee, E.M., Thomas, M.C., Huang, A.Y.C., Hauda, K.M.,  
40 Levitsky, H.I. and Pardoll, D.M. (1996), Journal of  
Immunotherapy 19 (3): 176-183
- Johnson, O.F., Cleland, J.L., Lee, H.J., Charnis, M.,  
Duenas, E., Jaworowicz, W., Shepard, D., Shahzamani,  
A., Jones, J.S., and Putney, S.D. (1996), Nature  
45 Medicine 2, 7: 795-799
- Kedar, E., Rutkowski, Y., Braun, E., Emanuel, N. and  
Barenholz, Y. (1994), J. Immunother. 16: 47-59
- Klibanov et al., (1990), FEBS Letters 268: 235



- Koppenhagen, F.J., (1997), Liposomes as delivery system for rekombinant interleukin-2 in anticancer immunotherapy, Thesis Utrecht Universitiy.
- Lamont, A.G., and Adorini, L. (1996). IL-12: a key cytokine in immune regulation. *Immunol Today* 5: 214-217
- Lee, H.J., et al., (1997), *J. Pharmacol. Exp. Ther.* 281(3): 1431-1439
- Lehmann, J.M., et al., (1989), *Proc. Natl. Acad. Sci. USA* 86, 9891-9895
- Longo, W.E. and Goldberg, E.P., (1985), *Methods Enzymol.* 112: 18-26
- Lowry, O.H., et al., (1951), *J. Biol. Chem.* 193: 265-275
- Marumo, K., et al., (1997), *Int. J. Urol.* 4(1): 55-61
- Maulding, H.V., (1987), *J. of Controlled Release* 6: 167-176
- Mayhew et al, (1992), *Int. J. Cancer* 51: 1-8
- Melief, C. JM. et al., (1996), *Current Opinion in Immunology* (8), 651 - 657
- Mellors, J.W., et al., (1989), *Infect. Immun.* 57(1): 132-137
- Mitchell, M.S., et al., (1993), *Ann. NY Acad. Sci.* 12; 690: 153-166
- Mori, A.; Klibanov, A. L.; Torchilin, V. P.; Huang, L., (1991), *FEBS Lett* 284: 263-6
- Morimoto, Y. and Fujimoto, S., (1985), *Crit. Rev. Ther. Drug Carrier Syst.* 2(1):19-36
- Morton, D.L., et al., (1989), *Semin Surg Oncol* 5(6): 420-425
- Morton, D., Foshag L., Hoon D., Nizze J., Famatiga E.m Wanek L., Chang C., Davtyan D., Gupta R. and Elashoff R. (1992), *Ann Surg* 216: 463-482
- Oratz, R., et al., (1989), *J Biol Response Mod* 8(4): 355-358
- Papahadjopoulos et al, (1991), *Proc. Natl. Acad. Sci. USA* 88: 11460-4
- Pardoll, D.M. (1995), *Ann. Rev. Immunol.* 13: 399-415.
- Perez, E.A., Scudder, S.A., Meyers, F.A., Tanaka, M.S., Paradise, C. and Gandara, D.R. (1991), *J. Immunother.* 10: 57-62
- Puccetti, P. et al., (1994), *Eur. J. Immunol.* 24, 1446-1452
- Rosenberg, SA. (1988). Immunotherapy of patients with advanced cancer using interleukin-2 alone or in combination with lymphokine activated killer cells. In *Important Advances in Oncology*, ed. V de Vita, S Hellman, SA Rosenberg, pp. 217-257. Philadelphia: JB Lippincott.

- Rosenberg, SA., Lotze, MT., Yang, JC., Aebersold, PA.,  
Lineham, WM., Seipp, CA., Withe, DE. (1989), Ann.  
Surg. 210: 474-485
- Rosenberg, S.A. (1991), Cancer Res. 51: 5074-5079.
- 5 Rosenberg, S.A., Anderson, W.F., Blaese, M.R.,  
Ettinghausen, S.E., Hwu, P. et al. (1992), Human  
Gene Therapy 3: 75-91
- Rosenstein, M., Ettighausen, S.E. and Rosenberg, S.A.  
(1986), J. Immunol. 137: 1735-1742
- 10 Saravolac, E.G., et al., (1996), Antiviral Res. 29(2-3):  
199-207
- Schmidt, W., Schweighoffer, T., Herbst, E., Maass, G.,  
Berger, M., Schilcher, F., Schaffner, G. and  
Birnstiel, M.L. (1995), Proc. Natl. Acad. Sci.  
15 USA 92: 4711-4714
- Schmidt, W., et al., (1996), Proc. Natl. Acad. Sci.  
USA 93(18): 9759-9763
- Schmidt, W., et al., (1997), Proc. Natl. Acad. Sci.  
USA 94(7): 3262-3267
- 20 Schweighoffer, T., Berger, M., Buschle, M., Schmidt, W.,  
and Birnstiel, M.L. (1996), Cytokines Mol. Ther. 2:  
185-192
- Senior, J.; Delgado, C.; Fisher, D.; Tilcock, C.;  
Gregoriadis, G., (1991), Biochim Biophys Acta 1062:  
25 77-82
- Tibbets, L.M., et al., (1993), Cancer, Jan. 15., Vol.71,  
2, 315-321
- Torchilin, V. P.; Klibanov, A. L.; Huang, L.; S, O. D.;  
Nossiff, N. D.; Khaw, B. A., (1992), Faseb J 6:  
30 2716-9
- Torchilin, V. P., et al., (1994), Biochim Biophys Acta  
1195: 181-184
- Torchilin, V. P., and Papisov, M. I., (1994), J Liposome  
Res 4(1): 725-739
- 35 van der Bruggen, PC., Traversari, C., Chomez, P.,  
Lurquin, C., DePlaen, E., Van den Eynde, B., Knuth,  
A., Boon, T. (1991), Science 264, 1643-1650
- Willmott, N., et al., (1989), J. Pharm. Pharmacol.  
41(7): 433-438
- 40 Woodle, M. C.; Newman, M. S.; Cohen, J. A., (1994), J  
Drug Target 2: 397-403
- Wölfel, T. et al., (1994) a), Int. J. Cancer 57, 413-418
- Wölfel, T. et al., (1994) b), Eur. J. Immunol. 24,  
759-764
- 45 Yoshioka, (1991), Biomaterials 12: 861-4
- Zatloukal, K., Schmidt, W., Cotten, M., Wagner, E.,  
Stingl, G. and Birnstiel, M.L. (1993), Gene 135:  
199-207

- [illegible]